

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/293825854>

# The Effect of Estradiol and Progesterone on Toll Like Receptor Gene Expression in A Human Fallopian Tube Epithelial Cell Line

Article in Cell Journal · February 2016

CITATIONS

9

READS

110

6 authors, including:



**Zahra Zandieh**

13 PUBLICATIONS 50 CITATIONS

[SEE PROFILE](#)



**Masoumeh Ashrafi**

University of Tabriz

4 PUBLICATIONS 18 CITATIONS

[SEE PROFILE](#)



**Alireza Fazeli**

The University of Sheffield

169 PUBLICATIONS 3,152 CITATIONS

[SEE PROFILE](#)



**Reza Aflatoonian**

Royan Institute

74 PUBLICATIONS 832 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



The role of TSGA10 in Spermatogenesis [View project](#)



Transplantation [View project](#)

# The Effect of Estradiol and Progesterone on *Toll Like Receptor* Gene Expression in A Human Fallopian Tube Epithelial Cell Line

Zahra Zandieh, D.V.M., Ph.D.<sup>1</sup>, Fatemehsadat Amjadi, M.Sc.<sup>1</sup>, Mahnaz Ashrafi, M.D.<sup>2</sup>, Abbas Aflatoonian, M.D.<sup>3</sup>, Alireza Fazeli, Ph.D.<sup>4</sup>, Reza Aflatoonian, M.D., Ph.D.<sup>5\*</sup>

1. Department of Anatomy, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

2. Department of Obstetrics and Gynecology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

3. Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

4. Academic Unit of Reproductive and Developmental Medicine, The University of Sheffield, Sheffield, United Kingdom

5. Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

\*Corresponding Address: P.O.Box: 16635-148, Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran  
Email: R.Aflatoonian@gmail.com

Received: 15/Apr/2014, Accepted: 21/Oct/2014

## Abstract

**Objective:** Toll like receptors (*TLRs*) are one of the main components of the innate immune system. It has been reported that expression of these receptors are altered in the female reproductive tract (FRT) during menstrual cycle. Here we used a fallopian tube epithelial cell line (OE-E6/E7) to evaluate the effect of two sex hormones in modulating *TLR* expression.

**Materials and Methods:** In this experimental study, initially *TLR* gene expression in OE-E6/E7 cells was evaluated and compared with that of fallopian tube tissue using quantitative real time-polymerase chain reaction (qRT-PCR) and immunostaining. Thereafter, OE-E6/E7 cells were cultured with different concentrations of estradiol and progesterone, and combination of both. qRT-PCR was performed to reveal any changes in expression of *TLR* genes as a result of hormonal treatment.

**Results:** *TLR1-10* genes were expressed in human fallopian tube tissue. *TLR1-6* genes and their respective proteins were expressed in the OE-E6/E7 cell line. Although estradiol and progesterone separately had no significant effect on *TLR* expression, their combined treatment altered the expression of *TLRs* in this cell line. Also, the pattern of *TLR* expression in preovulation (P), menstruation (M) and window of implantation (W) were the same for all *TLRs* with no significant differences between P, M and W groups.

**Conclusion:** These data show the significant involvement of the combination of estradiol and progesterone in modulation of *TLR* gene expression in this human fallopian tube cell line. Further experiments may reveal the regulatory mechanism and signalling pathway behind the effect of sex hormones in modulating *TLRs* in the human FRT.

**Keywords:** Estradiol, Progesterone, Fallopian Tube, Toll Like Receptors

Cell Journal(Yakhteh), Vol 17, No 4, Winter 2016, Pages: 678-691

**Citation:** Zandieh Z, Amjadi F, Ashrafi M, Aflatoonian A, Fazeli A, Aflatoonian R. The effect of estradiol and progesterone on toll like receptor gene expression in a human fallopian tube epithelial cell line. Cell J. 2016; 17(4): 678-691.

## Introduction

Infection within the upper regions of the female reproductive tract (FRT), particularly the fallopian tubes, can have serious consequences such as chronic pelvic inflammation, infertility and pregnancy complications (1, 2). For example, sexually

transmitted diseases (STDs) that infect the upper regions of the FRT are a major worldwide health problem (3, 4). Approximately 8% of females annually develop pelvic inflammatory disease (PID) and after re-infection, this risk increases by 40-70% (5). It is thus of paramount importance

that infectious agents are quickly recognised and removed from the upper parts of the FRT. Characterization of the defense systems present within the FRT will assist the development of effective therapies or vaccination strategies against STDs.

The innate immune system is the first line of defense against infection. This system is able to identify what is foreign or non-self and produces adequate responses that lead to the pathogens being suppressed. Toll like receptors (*TLRs*) are a family of pattern recognition receptors that recognise pathogen-associated molecular patterns (PAMP) and constitute a major part of the innate immune system (6, 7). Until now, eleven members of this receptor family have been discovered in humans. Of these, *TLR1-9* are conserved between human and mouse. Using various methods including ectopic expression of mammalian cDNA in cell lines, some of the activating ligands of *TLRs* have been discovered. Each individual *TLR* is known to detect molecules (ligands) from varying classes of microbial agents (8, 9). Some *TLRs* like *TLR2* and its associated receptors *TLR1* and *TLR6* mainly react against Gram-positive bacteria by detecting molecules from mycobacteria and Gram-positive bacteria (10-12). Some PAMP, like lipoteichoic acid (LTA), can be detected only by *TLR2* (11).

In contrast, *TLR1* associates with *TLR2* to recognize triacylated lipoproteins (13), whereas *TLR2* together with *TLR6* detects diacylated lipoproteins and peptidoglycans (14-16). *TLR4* recognises lipopolysaccharides (LPS) which are present in Gram-negative bacteria (17-19). *TLR5* recognises bacterial flagellin (20). Other *TLRs* mainly react against viruses. For example, *TLR3* recognises RNA from double stranded RNA viruses (21, 22). Also, *TLR7* and 8 recognise RNA from single stranded RNA viruses and antiviral compounds such as imidazoquinolines (16, 19, 23), whilst *TLR9* recognises unmethylated CpG DNA found richly in prokaryotic genomes and DNA viruses (19, 24). Like *TLR1* and *TLR6*, *TLR10* is another *TLR2*-associated receptor and also highly homologous to *TLR2*, however, the function of this *TLR* is not completely understood (25-27). In addition, it was revealed that the *TLR* pathway has physiological relevance to human fertility *TLR* by playing roles in ovulation, sperm capacitation, fertilization and pregnancy (28-31). They were also

shown to play a role in the pathophysiology of relevant disorders including endometriosis and poor ovarian response (32, 33).

Several studies have investigated the presence and the role of *TLRs* in the male and FRTs (34-43). Furthermore, the expression of *TLRs* in endometrial cell lines have been shown by Abussahoud et al. (44). It is evident that sex hormones modulate cells and their immune response potential, but varies throughout the FRT (45). Reports by us and others have demonstrated the existence of *TLRs* in FRT and the cycle-dependent expression of *TLRs* in the endometrium (34, 46). The effect of estradiol has also been showed in endometrial cells in several studies (47-49).

The cycle-dependent expression of *TLRs* in FRT implicates a role for sex hormones in regulation of *TLR* function in FRT. Here we used a fallopian tube epithelial cell line (OE-E6/E7) (50) to investigate the role of two sex hormones (estradiol and progesterone) in modulating *TLR* expression in the fallopian tube. This cell line has characteristics of human fallopian tube epithelial cells, including morphology, receptor expression and hormonal responses (50). Thereafter, the effect of the sex hormones and their combination as well as their antagonists on expression of *TLR1-10* in fallopian tube cells was investigated. This was done by testing *TLR* whether *TLR* expression is altered in the presence of sex hormones.

## Materials and Methods

### Fallopian tube tissue collection

This investigation was an experimental study approved by the Royan Institute Ethics Committee. Informed written consent was obtained prior to the collection of tissue samples. Human fallopian tube tissues were collected from 9 patients undergoing total abdominal hysterectomy for benign gynaecological conditions. The mean age of the women taking part in the study was 42 (range of 33-56) years with all in the secretory phase of their menstrual cycle. For genomic studies, fallopian tube tissue samples were immediately placed in RNAlater (Ambion, UK) and stored for 24 hours at 4°C followed by immersion and storage in liquid nitrogen until the time of processing.

### Antibodies and peptides

Antibodies and peptides used in the experiments were obtained from Santa Cruz Biotechnology Inc. (USA). These were goat polyclonal antibodies specific for N-terminal domains of *TLR1*, *TLR2*, *TLR3*, *TLR5* and *TLR6*, and a goat polyclonal antibody specific for the C-terminal domain of *TLR4*. Blocking peptides specific for the respective antibodies were used to detect non-specific staining.

### Immunostaining

For immunostaining, OE-E6/E7 cells were cultured in four well chamber slides. They were cultured at 37°C in Dulbecco's Modified Eagle Medium- F12 (DMEM-F12) culture medium (Invitrogen, UK) supplemented with 1% penicillin and streptomycin (Sigma-Aldrich, UK), 10% fetal calf serum (FCS, Invitrogen, USA) and L-glutamine (Invitrogen, USA) in 5% CO<sub>2</sub> atmosphere. At confluency, the slides were washed five times with Ca<sup>2+</sup> and Mg<sup>2+</sup> free phosphate buffered saline (PBS, Gibco, USA), fixed with 5% formalin and stored at 4°C until use.

Formalin-fixed slides were washed in PBS and then stained using a Vectastain Elite ABC peroxidase kit (Vector Laboratories Ltd, UK). In addition, to avoid non-specific binding, an avidin/biotin blocking kit (Vector) was used. Briefly, slides were blocked for 1 hour at room temperature in PBS solution containing 0.2% v/v horse serum and 25% v/v avidin supplied in the blocking kit. The block was subsequently removed and slides were incubated for 2 hours at room temperature with primary antibody at an appropriate dilution using antibody diluent media (Dakocytomation Ltd, UK) and 250 µl biotin per ml of diluted antibody. Binding was then visualized by incubating the slide with peroxidase substrate 3-amino-9-ethylcarbazole (AEC) (Vector) for 10 minutes, washed in distilled water for 3 minutes and counterstained in 10% haematoxylin for 10 minutes. Slides were finally washed in tap water for 2 minutes and mounted with Aquamount (VWR International, UK).

Optimal staining was achieved by incubating slides with different concentrations of *TLR* antibodies (*TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR6* with 4, 4, 10, 10, 4 and 10 µg/ml respectively).

Negative control sections were obtained by blocking the primary antibody with the corresponding specific peptide. Immunostained sections were examined using an Olympus BH2 microscope (Olympus, UK).

### Toll like receptors expression in OE-E6/E7 cells

*TLR* expression in the OE-E6/E7 cell line was investigated and compared to fallopian tube tissue samples. OE-E6/E7 cells were cultured at 37°C in DMEM (F12) supplemented with 1% penicillin and streptomycin, 10% FCS and L-glutamine in 5% CO<sub>2</sub> atmosphere. At confluency, cells were washed with Ca<sup>2+</sup> and Mg<sup>2+</sup> free PBS and then harvested using trypsin-Ethylenediaminetetraacetic acid (EDTA, Invitrogen, USA) pelleted by centrifugation at 300 g for 5 minutes. One ml of TRI-reagent (Sigma, UK) was added onto the pellet (5×10<sup>6</sup> cells). Thereafter, total RNA from pelleted cells was extracted following the standard protocol supplied by the manufacturer.

On the day of the experiment, fallopian tube tissues were removed from RNAlater and homogenised in 3 ml of TRI reagent using an Ultra-Turrax homogenizer for 2 minutes following the standard protocol supplied by the manufacturer.

Total RNA obtained from OE-E6/E7 cells and fallopian tube tissue samples (by using chloroform and isopropanol) were then treated with DNase I (DNA-free™, Ambion, USA) to remove genomic DNA contamination from the samples. First strand cDNA was synthesized by reverse transcription using oligodT primers (Metabion, Germany) and SuperScript II (200 U/µl, Invitrogen, USA). RT controls were prepared without the enzyme (non-reverse-transcribed controls).

Polymerase chain reaction (PCR) was performed using prepared cDNA, Platinum Blue PCR Super Mix (Invitrogen, USA) and primers from Metabion (Table 1). The amplification was run for 40 cycles under the following conditions: initial heat at 95°C for 30 seconds, 59°C to 65°C for 30 seconds and final annealing at 72°C for 30 seconds. All experiments included reverse transcriptase (RT) controls as well as negative controls (no cDNA). PCR products were visualised on a 1.2 % agarose gel. All amplified PCR products were sequenced to confirm the identity of the amplified product.

**Table 1:** Sequence of primers used in this study

Genes	Primer (5'-3')	Annealing temperature (C)	Accession no.	Product size (bp)	Reference
<i>TLR1</i>	F: GGGTCAGCTGGACTTCAGA R: AAAATCCAAATGCAGGAACG	63	Gene Bank: U88540.1	250	43
<i>TLR2</i>	F: TCGGAGTTCTCCCAGTTCTCT R: TCCAGTGCTTCAACCCACAA	60	Gene Bank: NM_003264.3	175	43
<i>TLR3</i>	F: GTATTGCCTGGTTTGTAAATTGG R: AAGAGTTCAAAGGGGGCACT	60	Gene Bank: NM_003265.2	156	43
<i>TLR4</i>	F: TGATGTCTGCCTCGCGCCTG R: AACCACTCCACGCAGGGCT	60	Gene Bank: NM-138554.3	98	32
<i>TLR5</i>	F: CACCAAACCAGGGATGCTAT R: CCTGTGTATTGATGGGCAAA	60	Gene Bank: NM_003268.5	111	43
<i>TLR6</i>	F: GCCACCATGCTGGTGTGGCT R: CGCCGAGTCTGGGTCCACTG	60	Gene Bank: NM-006068.4	101	43
<i>TLR7</i>	F: CCTTGAGGCCAACAACATCT R: GTAGGGACGGCTGTGACATT	63	Gene Bank: NM_016562.3	285	43
<i>TLR8</i>	F: CTTCGATACCTAAACCTCTCTAGCAC R: AAGATCCAGCACCTTCAGATGA	60	Gene Bank: NM_138636.4	90	43
<i>TLR9</i>	F: TTCCCTGTAGCTGCTGTCC R: ACAGCCAGTTGCAGTTCACC	60	Gene Bank: NM_017442.3	207	43
<i>TLR10</i>	F: TGCCCACCACAATCTCTTCCATGA R: AGCAGCTCGAAGGTTTGCCCA	60	Gene Bank: NM-030956.3	184	43
<i>β-actin</i>	F: CAAGATCATTGCTCCTCCTG R: ATCCACATCTGCTGGAAGG	60	Gene Bank: NM-001101	90	43
<i>GAPDH</i>	F: CTCATTTCTGGTATGACAACGA R: CTCCTCTTGTGCTCTTGCT	60	Gene Bank: NM_002046.4	122	43

TLR; Toll like receptor.



### Cell culture in the presence of sex hormones

To investigate the effect of estradiol and progesterone on *TLR* expression in the OE-E6/E7 cell line, OE-E6/E7 cells were cultured again in triplicates at 37°C in DMEM (F12) culture medium and water soluble estradiol and progesterone (Sigma-Aldrich, UK) to reach the final concentrations of 0.1, 1, 10, 100 nM and 1, 10, 100, 1000 nM for estradiol and progesterone respectively. In addition, the following combinations of these two (final concentrations) were used in four groups of: control (C, without any additional treatment of sex hormones), menstruation (M, 1 nM progesterone and 0.1 nM estradiol), pre-ovulation (P, 6.5 nM progesterone and 1.5 nM estradiol) and window of implantation (W, 35 nM progesterone and 1 nM estradiol) in 5% CO<sub>2</sub> atmosphere in 75 ml flasks for 24 hours in the absence of phenol red and serum. In the next step, the effect of sex hormone antagonists on *TLR* expression was evaluated. To do this, OE-E6/E7 cells were divided in two groups. One group was pre-treated for 2 hours with 1 µM ICI 182, 780 (fulvestrant, an estradiol antagonist, Sigma-Aldrich, UK) and the other pre-treated for 2 hours with 0.1 µM RU486 (mifepristone, a progesterone antagonist, Tocris, USA). After pre-treatment, both groups were treated again separately with a combination of estradiol and progesterone based on the four treatment groups (C, M, P and W) in 5% CO<sub>2</sub> atmosphere in 75 ml flasks for 24 hours in the absence of phenol red and serum.

Cells were next treated for RNA isolation and cDNA synthesis following the same protocol mentioned above. qRT-PCR was performed using the cDNA prepared from the estradiol and progesterone treatment experiments, same primers as in table 1 and SYBR Green Jump Start (Sigma, UK) master mix (containing 10 µl SYBR Green, 7 µl Water, 1 µl of each primer and 1 µl cDNA). The PCR amplification was performed under the following conditions: 50 cycles of 95°C for 30 seconds, 59°C to 63°C for 30 seconds and 72°C for 30 seconds. All experiments included RT controls and negative controls (no cDNA). qRT-PCR was performed on a Mx3005P QPCR machine (Stratagene, Germany) and results were analyzed using MxPro QPCR software version 4.01. In preliminary experiments, the efficiency of the primer sets of each Q-PCR reaction was established. Variation in *Beta-actin* and *GAPDH* expression as two

housekeeping genes was also tested.

The qRT-PCR data were analyzed using the comparative CT method (51). The fold change was calculated as  $FC = 2^{-\Delta\Delta CT}$ .

The results were expressed as mean ± SEM. Significance testing was performed by one-way ANOVA with Tukey's multiple comparison test.  $P < 0.05$  was considered significant.

## Results

### Reverse transcriptase-polymerase chain reaction

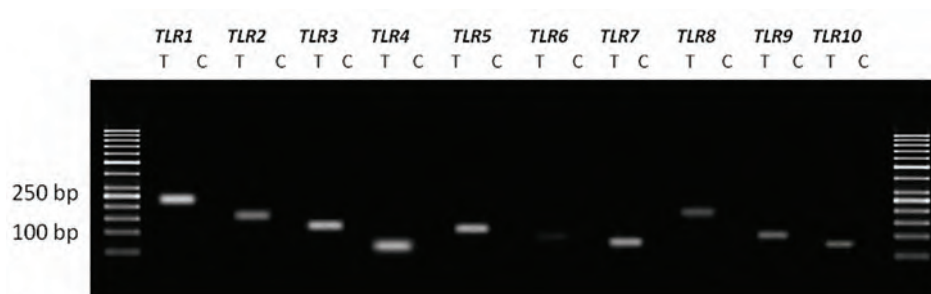
Figure 1 shows the results of RT-PCR of *TLR1-10* genes in the human fallopian tube tissue. Figure 2 shows the results of RT-PCR of *TLR1-6* genes in OE-E6/E7 cells. Size of all amplified PCR products were as predicted and sequencing verified correct amplification of each gene. No product was amplified in negative control samples, indicating absence of genomic DNA contamination.

### Immunostaining

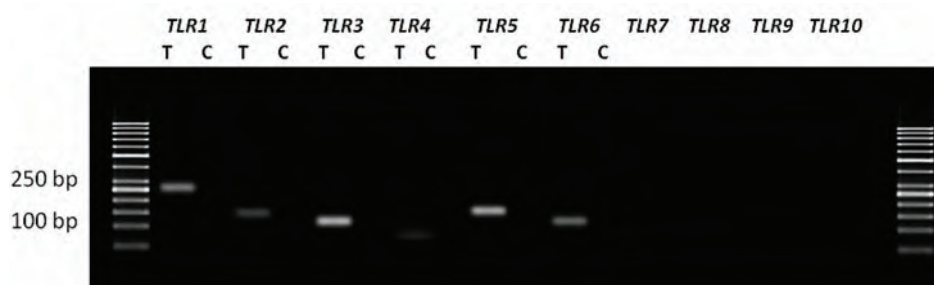
Formalin-fixed slides were used to study the distribution of *TLR1-6* in OE-E6/E7 cells. Positive immunostaining for all six *TLRs* was observed with *TLR1*, 2, 4 and *TLR6* displaying moderate staining in this epithelial cell line. However, strong staining for *TLR3* and *TLR5* was observed. The immunocytochemical localisation of *TLR1-6* is shown in figure 3.

### Quantitative polymerase chain reaction

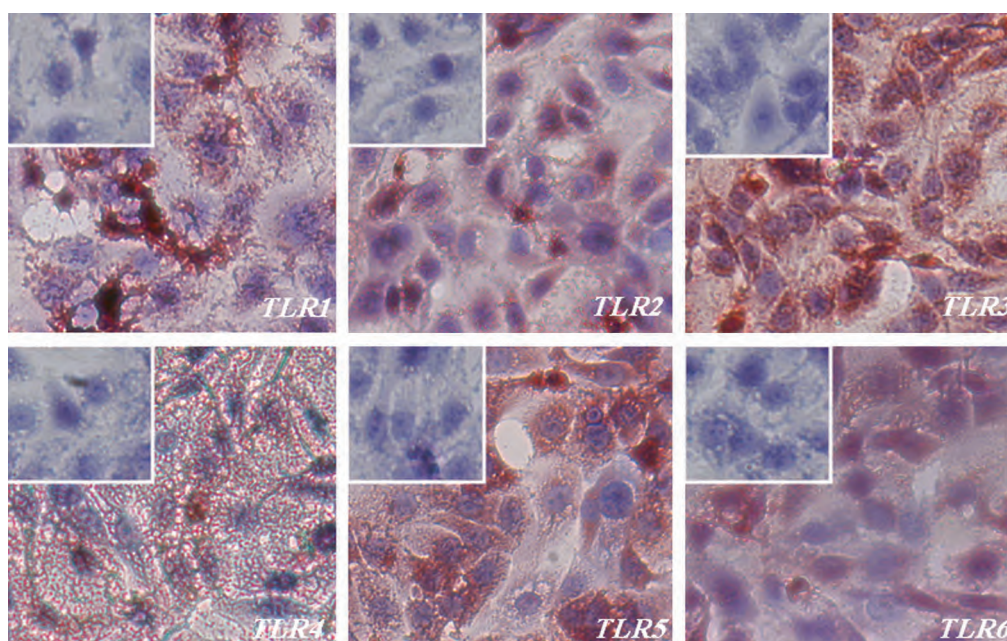
A stable expression of *Human β-actin* and *GAPDH* genes with variable hormonal treatments was observed (Fig.4). The quantitative expression profiles of *TLR1-6* genes in OE-E6/E7 cells treated with varying concentrations of estradiol and progesterone are shown in figures 5 and 6 respectively. The relative expression of *TLR1-6* genes did not significantly differ in response to different concentrations of either estradiol or progesterone. The mean relative expression of *TLR1-6* differed considerably between P, M, W and C groups (Fig.7). The Pattern of *TLR* expression in P, M and W were significantly different among P, M and W groups. Also, by using 1 µM of fulvestrant (estradiol antagonist) or 0.1 µM mifepristone (progesterone antagonist) 2 hours prior to the combined estradiol and progesterone treatment, the mean relative expression of *TLR1-6* did not differ markedly in C, P, M and W groups (Figs.8, 9).



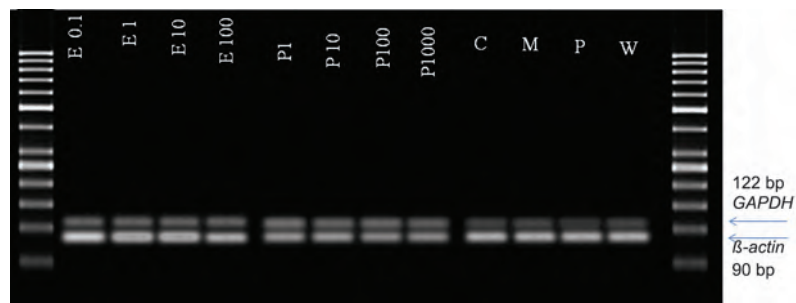
**Fig.1:** The expression of *TLR1-10* genes in the human fallopian tube tissue. Each pair of primers produced a specific product with the specific predicted size observed in the test (T) samples. C; Control samples (samples without using cDNA) and *TLR*; *Toll like receptor*.



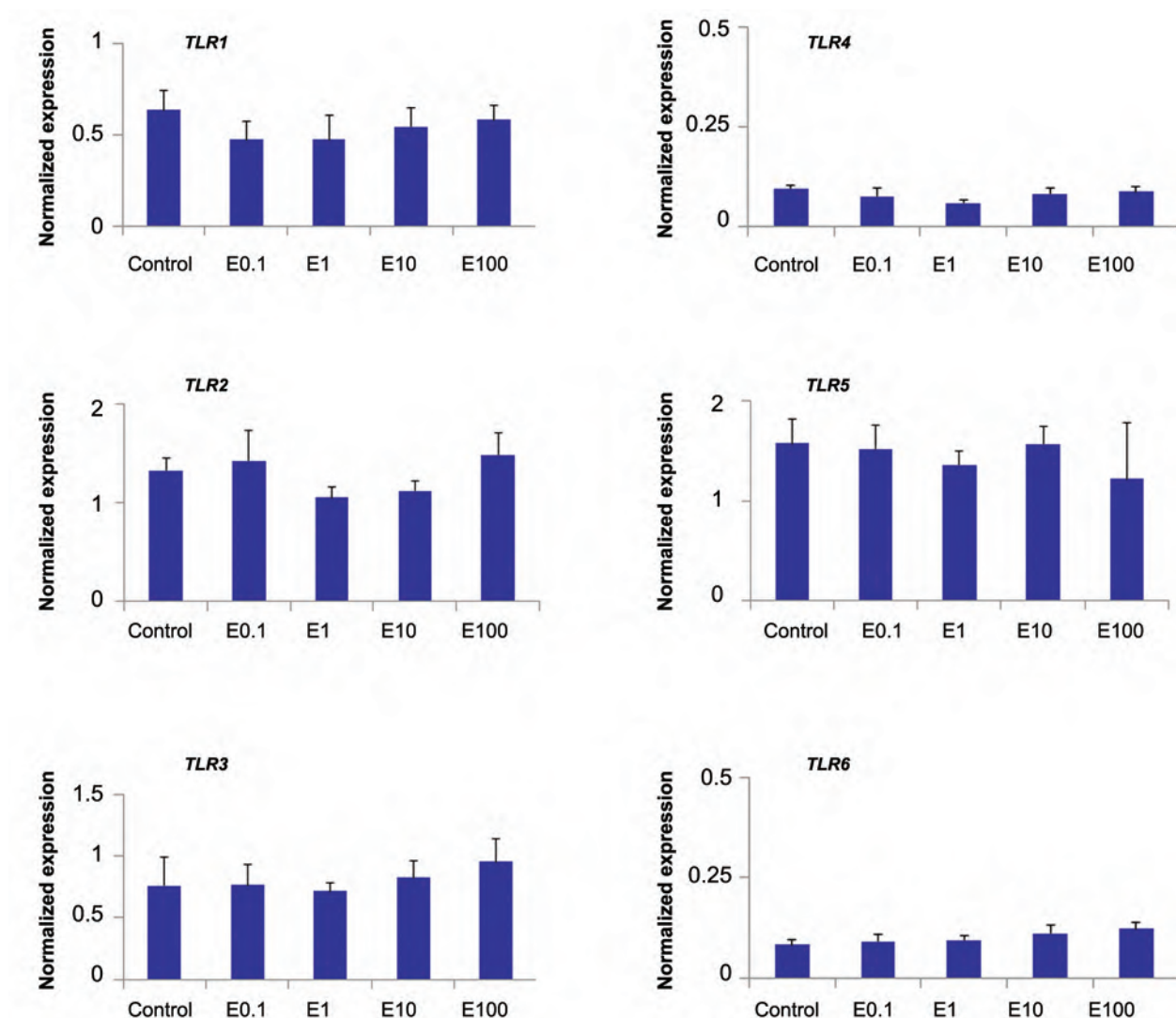
**Fig.2:** The expression of *TLR1-6* genes in the human fallopian tube cell line (OE-E6/E7). Each pair of primers produced a specific product with the specific predicted size observed in the test (T) samples. C; Control samples (samples without using cDNA) and *TLR*; *Toll like receptor*.



**Fig.3:** Immunohistochemical staining of *TLR1-6* in the OE-E6/E7 cell line. Positive staining is red, negative staining is blue. Small inserts show blocking of anti *TLR1-6* antibodies with respective specific peptides. *TLR*; *Toll like receptor*.

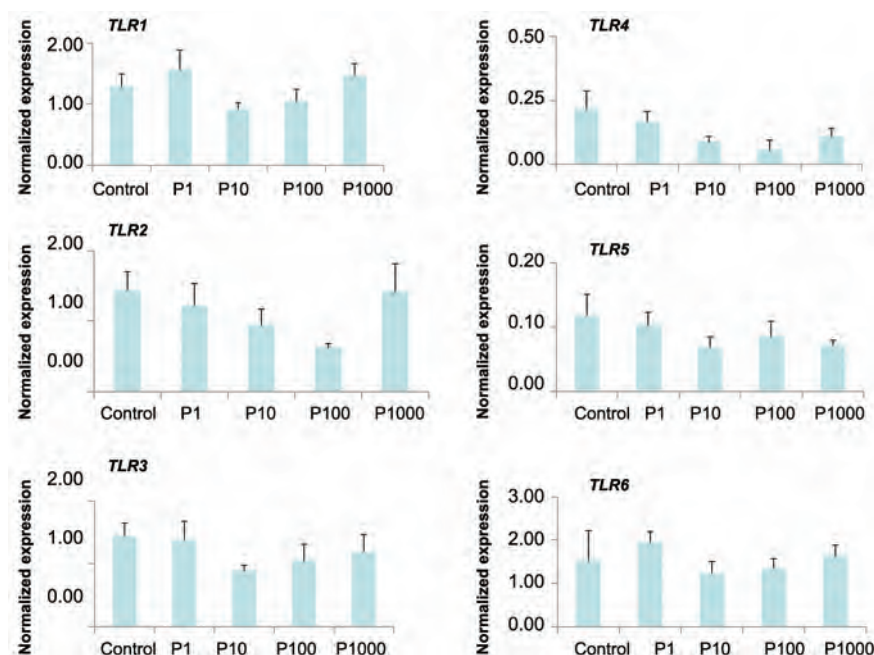


**Fig.4:** The stable expression of *GAPDH* and  $\beta$ -*actin* in OE-E6/E7 under variable hormonal treatments. **E**; Estradiol, **P**; Progesterone, **C**; Control, **M**; Menstruation and **W**; Window of implantation.

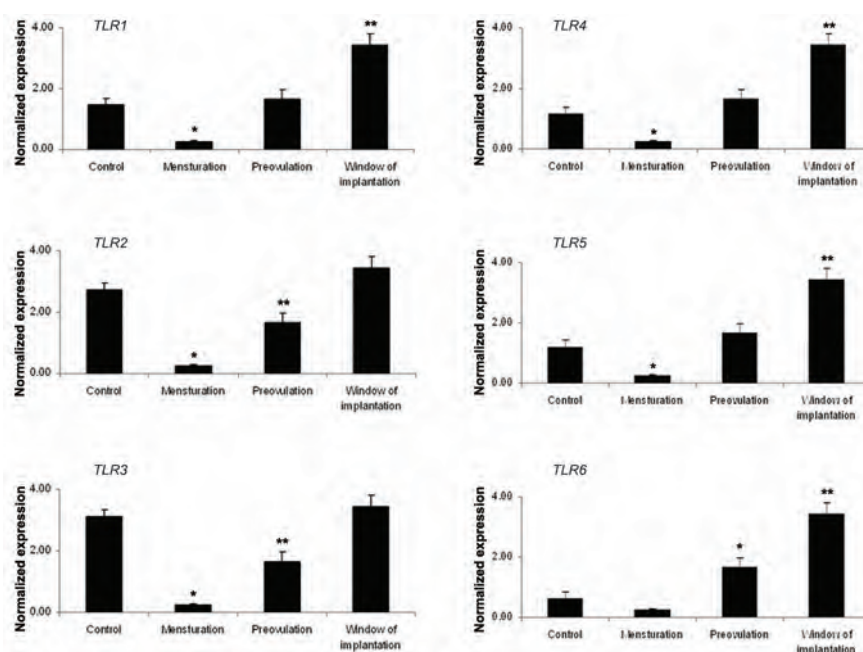


**Fig.5:** Expression of *TLR1-6* in OE-E6/E7 cultured with estradiol. Mean  $\pm$  SEM of normalized expression values (with  $\beta$ -*actin*) for *TLR1-6* genes in OE-E6/E7 cultured with different concentrations of estradiol (control: 0 nM, E 0.1: 0.1 nM, E1: 1 nM, E10: 10 nM and E100: 100 nM or 1  $\mu$ M). No significant results were obtained. *TLR*; Toll like receptor.

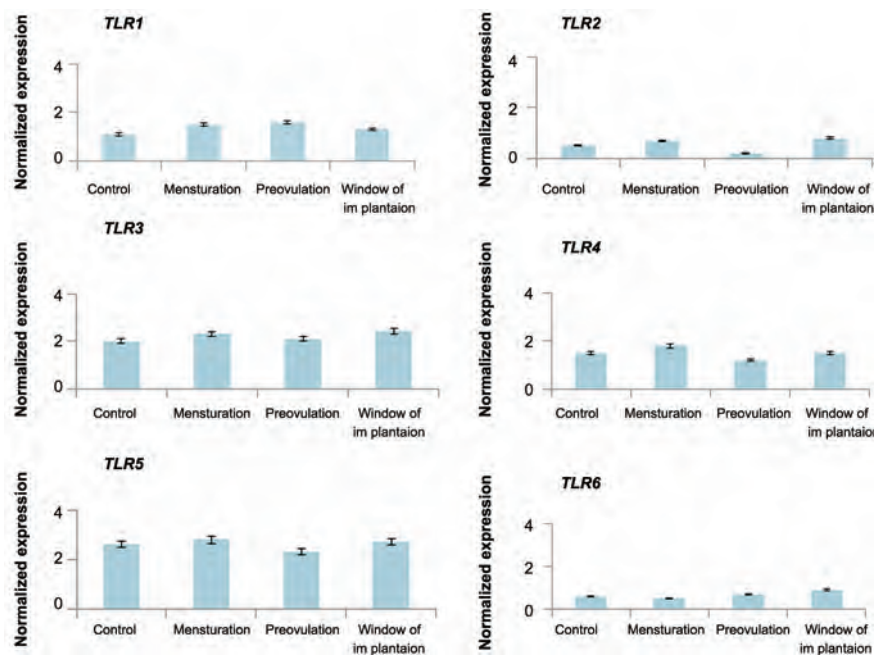




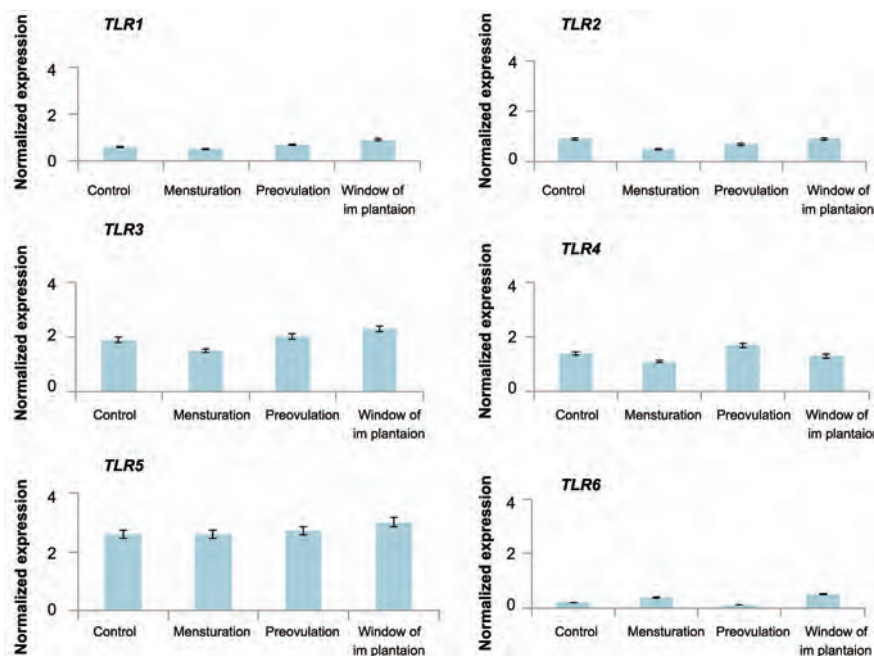
**Fig.6:** Expression of *TLR1-6* in OE-E6/E7 cultured with progesterone. Mean  $\pm$  SEM of normalized expression values (with  $\beta$ -actin) for *TLR1-6* genes in OE-E6/E7 cultured with different concentrations of progesterone (control: 0 nM, P1: 1 nM, P10: 10 nM, P100: 100 nM and P1000: 1000 nM or 1  $\mu$ M). No significant results were obtained. *TLR*; Toll like receptor.



**Fig.7:** Expression of *TLR1-6* in OE-E6/E7 cultured with a combination of estradiol and progesterone concentrations. Mean  $\pm$  SEM of normalized expression values (with  $\beta$ -actin) for *TLR1-6* genes in OE-E6/E7 cultured with a combination of estradiol and progesterone. Control (C, without any additional treatment of sex hormone), menstruation (M, 1 nM progesterone and 0.1 nM estradiol), pre-ovulation (P, 6.5 nM progesterone and 1.5 nM estradiol) and window of implantation (W, 35 nM progesterone and 1 nM estradiol) (control: 0 nM, P1: 1 nM, P10: 10 nM, P100: 100 nM and P1000: 1000 nM or 1  $\mu$ M). Star denotes statistically significant differences. *TLR*; Toll like receptor.



**Fig.8:** Expression of *TLR1-6* in OE-E6/E7 precultured with fulvestrant, then cultured with a combination of estradiol and progesterone concentrations. Mean  $\pm$  SEM of normalized expression values (with  $\beta$ -actin) for *TLR1-6* genes in OE-E6/E7 pre-cultured with fulvestrant for 2 hours, then cultured with a combination of estradiol and progesterone in 4 groups (C, M, P, W). No significant results were obtained. *TLR*; Toll like receptor.



**Fig.9:** Expression of *TLR1-6* in OE-E6/E7 pre-cultured with mifepristone and cultured with a combination of estradiol and progesterone in 4 groups (C, M, P, W). Mean  $\pm$  SEM of normalized expression values (with  $\beta$ -actin) for *TLR1-6* genes. No significant results were obtained. *TLR*; Toll like receptor.

## Discussion

Epithelial cells are the first layer of defense against pathogens ascending FRT. Considering their role in protecting the tract from the external environment, *TLRs* are expected to be present in this tissue. Several studies have investigated the presence and the role of *TLRs* in FRT especially within the fallopian tube (34-42, 52). Previously, Pioli et al. (39), showed the presence of *TLR1-6* in the FRT as well as the accessory molecule CD14 and the molecular adapter MyD88 both of which are needed for *TLR* signalling. They also observed expression of *TLR1-6* at the transcript level in fallopian tubes. In agreement with this report, we previously showed the localization of *TLR1-6* in the fallopian tube using immunohistochemistry (36). Both observations are in agreement with our findings in this study that demonstrate the presence of *TLR1-6* transcripts in human fallopian tube tissues and cell line. The expression of *TLR4* protein and transcript has been detected in human endometrial epithelial cells, stromal cells (38) and fallopian tube stromal fibroblasts (53). However, *TLR4* expression was not detected in fallopian tube epithelial cells by Itoh et al. (53). In another study Hart et al. (42), reported the expression of *TLR7-9* in the fallopian tube, uterine, endometrium, cervix and ectocervix, while *TLR10* expression was restricted to the fallopian tube.

In this study, although expression of *TLR1-10* was detected in human fallopian tube tissues, expression of only *TLR1-6* was detected in OE-E6/E7 cells. This may be due to difference in features of the fallopian tube tissue and this specific cell line. Fallopian tube tissue contains an epithelial layer, stroma and capillaries supplying blood. Within the epithelial layer of the fallopian tube tissue, some of the cells are ciliated and some are known as secretory cells. The OE-E6/E7 cell line is only an isolated fallopian tube epithelial layer cell line. Therefore, the expression of *TLR* genes and their level of expression in this cell line is likely to be different compared with the original tissue consisting of different types of cells. The other potential explanation for the absence of expression of some *TLRs* in OE-E6/E7 cells may be due to differences between immortalised cell lines and their original parental primary cells. It is known that cell lines may undergo changes due to the process of immortalization. However, the characteristics

of this immortalized cell line have been compared to the parental human fallopian tube epithelium in several investigations. For example, human oviduct-specific glycoprotein, estradiol receptors and cytokeratin molecules have been found to be produced in both primary fallopian tube cells and OE-E6/E7 cells (50, 54, 55).

The FRT environment is under the control of sex hormones during the menstrual cycle. Sex hormones not only regulate anatomical and histological characteristics of this tract (56, 57), they are also involved in the influx and localization of immune cells in this tract (58-60). For example, uterine natural killer (uNK) cells are found in the human uterus in large numbers and are spread throughout the endometrium with increasing numbers as the menstrual cycle progresses (61, 62). In addition, the adaptive immune system is also influenced by the changing levels of sex hormones during the menstrual cycle. Antigen presentation has been shown to be suppressed in response to increasing concentrations of estradiol (63, 64). Sex hormones including estradiol and progesterone tightly control the distribution of macrophages within the endometrium (65-68). Another study demonstrated that estradiol, which is secreted by the ovary during the menstrual cycle, modulates epithelial cells and other immune cells in FRT directly and indirectly to regulate a wide range of immune functions specific to each site in FRT (69). Nasu and Narahara (49) also showed that antigen-presenting cells in the uterus and vagina are responsive to estradiol where antigen presentation as well as co-stimulatory molecule expression is inhibited by estradiol. Furthermore, they suggested that antigen-presenting cells in the uterus and vagina respond to selected *TLR* agonists with altered antigen presentation.

It is therefore likely that the action of *TLRs* is also modified by changing concentrations of estradiol and progesterone. Although all *TLR* molecules are expressed throughout the cycle, the majority of these genes are expressed at their lowest level during menstrual and proliferative stages of the cycle (34). At follicular stage of the cycle, while progesterone levels are at their lowest, estradiol levels are at their highest. This may indicate an inhibitory effect of estradiol and/or enhancing influence of progesterone on the expression of *TLR* molecules in FRT, especially in the endometrium.

It is plausible that this alteration in *TLR* gene expression may even influence the function of *TLRs* in mediating innate immune responses in FRT. To test these hypotheses, we examined the effect of sex hormones on *TLR* expression in the OE-E6/E7 cell line. We used various concentrations of estradiol and progesterone to determine the effects of these sex hormones on *TLR* expression. No significant alteration in the relative expression of *TLR1-6* transcripts was observed in the fallopian tube cell line in response to different concentrations of estradiol and progesterone. These results agreed with the findings of Lesmeister et al. (47) who also showed that *in vitro* treatment of endometrial epithelial cell line (RL95-2) with 17 $\beta$ -estradiol did not have an effect on *TLR3* transcript or protein expression. Also, in a recent study on human uterine epithelial cells and the ECC-1 uterine epithelial cell line, Nasu and Narahara (49) demonstrated that estradiol either alone or prior to treatment with poly (I:C), had no effect on the expression of interferon  $\beta$  (IFN $\beta$ ) or interferon-stimulated genes (ISG). However, another study showed that production and secretion of protective antimicrobials including human  $\beta$  defensin-2 (HBD2) and secretory leukocyte protease inhibitor (SLPI) are directly upregulated by estradiol. On the contrary, estradiol inhibited LPS and poly (I:C)-induced secretion of macrophage inhibitory factor (MIF), interleukin 6 (IL-6) and IL-8 in primary uterine epithelial cells (70).

In the next step of our study, combined effect of estradiol and progesterone was evaluated. Expression of *TLR1-6* was higher when the level of sex hormones in culture media was comparable to their concentration in serum during the window of implantation (group W) in the menstrual cycle. These results agreed with our previous findings where higher expression of *TLRs* was observed during the secretory phase in human endometrium (34, 71). These findings demonstrated a stimulatory effect of co-presence of both sex hormones (M, P and W) on *TLR1-6* expression.

A recent study reported the fluctuation of *TLR* responsiveness in peripheral blood throughout the menstrual cycle (72). In agreement with our data, during the follicular phase, lower levels of IL-6 and tumor necrosis factor (TNF)- $\alpha$  following stimulation with the *TLR2* agonist, lower levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  following stimulation with

the *TLR4* agonist LPS, and lower levels of IL-1 $\beta$  and TNF- $\alpha$  following stimulation of whole blood with the *TLR5* agonist flagellin was observed when compared with the early luteal phase (72). Jorgenson et al. (46) also illustrated that *TLR3* transcript level in primary endometrial epithelial cells is menstrual cycle-dependent. They showed *TLR3* was expressed throughout the menstrual cycle but at its highest during the secretory phase of the cycle. In another study, Yao et al. (73) reported that except *TLR11*, the expression of *TLR1-10* is cycle-dependent in mouse. Whether the above mentioned findings are due to estradiol, progesterone or their combined effect on FRT epithelium remains to be deciphered.

When fulvestrant (estradiol antagonist) or mifepristone (progesterone antagonist) was used, the combined effect of estradiol and progesterone on *TLR* expression was inhibited. These data thus confirm our results that on the expression of *TLR1-6* is not affected by progesterone and estradiol individually *TLR1-6* but *TLR1-6* under the synergistic/additive effect of the combination of estradiol and progesterone.

In performing this investigation, we took several precautionary measures. The effect of hormones in cell culture experiments was tested in the absence of phenol red and serum. Phenol red has estradiolic properties and serum may contain small molecules with estradiolic effects and if present, may hamper the results of experiments.

In *in vitro* culture systems and particularly in the presence of blood or serum samples, progesterone degrades quickly (74). To avoid early degradation of hormones during our cell culture experiments, we used water soluble estradiol and progesterone (Cyclodextrin-encapsulated 17 $\beta$ -estradiol and progesterone) which are stable and tested for cell culture applications. These compounds have been used in several similar investigations (75-79). Furthermore, our results clearly demonstrated that these compounds do not alter *TLR* gene expression in cultured cells.

It seems that the pattern of *TLR* expression under different concentrations of estradiol and progesterone (mimicking those of the menstrual cycle) is similar to the pattern of *TLR* expression in endometrial tissue during the menstrual cycle (34). On the other hand, higher expression of *TLRs* in the



proliferative phase compared with menstruation as well as their higher expression in the secretory phase than other phases show that safety in the human fallopian tube at the time of ovulation or early embryo development is a key factor.

Future studies should be directed towards understanding the role of signalling pathways that enable estradiol and progesterone to modulate the expression and function of *TLRs* in FRT.

## Conclusion

This study firmly points to the involvement of sex hormones in modulation of *TLR* gene expression in human fallopian tube cells. Further experiments should be undertaken to reveal the regulatory mechanism(s) and signalling pathway(s) responsible for the effect of sex hormones in modulating innate immunity in human FRT.

## Acknowledgments

This study was financially supported by Royan Institute. We wish to thank Dr Kai-Fai Lee for his kind courtesy (providing us with the OE-E6/E7 cell line) and Miss Arghavan Janan for skillful technical assistance. The authors declare that they have no conflict of interest.

## References

- McCormack WM. Pelvic inflammatory disease. *N Engl J Med*. 1994; 330(2): 115-119.
- Witkin SS. Immunological aspects of genital chlamydia infections. *Best Pract Res Clin Obstet Gynaecol*. 2002; 16(6): 865-874.
- Cates W Jr. Priorities for sexually transmitted diseases in the late 1980s and beyond. *Sex Transm Dis*. 1986; 13(2): 114-117.
- Piot P, Plummer FA, Mhalu FS, Lamboray JL, Chin J, Mann JM. AIDS: an international perspective. *Science*. 1988; 239(4840): 573-579.
- Jiang J, Karimi O, Ouburg S, Champion CI, Khurana A, Liu G, et al. Interruption of CXCL13-CXCR5 axis increases upper genital tract pathology and activation of NKT cells following chlamydial genital infection. *PloS One*. 2012; 7(11): e47487.
- Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol*. 2001; 2(8): 675-680.
- Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol*. 2005; 17(1): 1-14.
- Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002; 20: 197-216.
- Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003; 21: 335-376.
- Medzhitov R, Janeway C Jr. The Toll receptor family and microbial recognition. *Trends Microbiol*. 2000; 8(10): 452-456.
- Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem*. 1999; 274(25): 17406-17409.
- Underhill DM, Ozinsky A, Smith KD, Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc Natl Acad Sci USA*. 1999; 96(25): 14459-14463.
- Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol*. 2002; 169(1): 10-14.
- Nakao Y, Funami K, Kikkawa S, Taniguchi M, Nishiguchi M, Fukumori Y, et al. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *J Immunol*. 2005; 174(3): 1566-1573.
- Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA*. 2000; 97(25): 13766-13771.
- Wetzler LM. The role of Toll-like receptor 2 in microbial disease and immunity. *Vaccine*. 2003; 21 Suppl 2: S55-60.
- Akashi S, Nagai Y, Ogata H, Oikawa M, Fukase K, Kusumoto S, et al. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. *Int Immunol*. 2001; 13(12): 1595-1599.
- Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol*. 2002; 3(7): 667-672.
- Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect*. 2004; 6(15): 1382-1387.
- Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 2001; 410(6832): 1099-1103.
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. 2001; 413(6857): 732-738.
- Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem*. 2004; 279(13): 12542-12550.
- Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol*. 2002; 3(6): 499.
- Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature*. 2000; 408(6813): 740-745.
- Flacher V, Bouschbacher M, Verronese E, Massacrier C, Sisrak V, Berthier-Vergnes O, et al. Human langerhans cells express a specific TLR profile and differentially respond to viruses and gram-positive bacteria. *J Immunol*. 2006; 177(11): 7959-7967.
- Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol*. 2005; 174(5): 2942-2950.
- Chuang T, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. *Biochim Biophys Acta*. 2001; 1518(1-2): 157-161.
- Growe RG, Luster MI, Fail PA, Lippes J. Quinacrine-induced occlusive fibrosis in the human fallopian tube is due to a unique inflammatory response and modification

- of repair mechanisms. *J Reprod Immunol*. 2013; 97(2): 159-166.
29. Ghosh M, Schaefer TM, Fahey JV, Wright JA, Wira CR. Antiviral responses of human Fallopian tube epithelial cells to toll-like receptor 3 agonist poly(I:C). *Fertil Steril*. 2008; 89(5 Suppl): 1497-1506.
30. Koga K, Aldo PB, Mor G. Toll-like receptors and pregnancy: trophoblast as modulators of the immune response. *J Obstet Gynaecol Res*. 2009; 35(2): 191-202.
31. Amirchaghmaghi E, Taghavi SA, Shapouri F, Saeidi S, Rezaei A, Aflatoonian R. The role of Toll like receptors in pregnancy. *Int J Fertil Steril*. 2013; 7(3): 147-153.
32. Taghavi SA, Ashrafi M, Mehdizadeh M, Karimian L, Joghataie M, Aflatoonian R. Toll-like receptors expression in follicular cells of patients with poor ovarian response. *Int J Fertil Steril*. 2014; 8(2): 183-192.
33. Khan KN, Kitajima M, Fujishita A, Nakashima M, Masuzaki H. Toll-like receptor system and endometriosis. *J Obstet Gynaecol Res*. 2013; 39(8): 1281-1292.
34. Aflatoonian R, Tuckerman E, Elliott SL, Bruce C, Aflatoonian A, Li TC, et al. Menstrual cycle-dependent changes of Toll-like receptors in endometrium. *Hum Reprod*. 2007; 22(2): 586-593.
35. Andersen JM, Al-Khairi D, Ingalls RR. Innate immunity at the mucosal surface: role of toll-like receptor 3 and toll-like receptor 9 in cervical epithelial cell responses to microbial pathogens. *Biol Reprod*. 2006; 74(5): 824-831.
36. Fazeli A, Bruce C, Anumba DO. Characterization of Toll-like receptors in the female reproductive tract in humans. *Hum Reprod*. 2005; 20(5): 1372-1378.
37. Fichorova RN, Cronin AO, Lien E, Anderson DJ, Ingalls RR. Response to *Neisseria gonorrhoeae* by cervicovaginal epithelial cells occurs in the absence of Toll-like receptor 4-mediated signaling. *J Immunol*. 2002; 168(5): 2424-2432.
38. Hirata T, Osuga Y, Hirota Y, Koga K, Yoshino O, Harada M, et al. Evidence for the presence of Toll-like receptor 4 system in the human endometrium. *J Clin Endocrinol Metab*. 2005; 90(1): 548-556.
39. Pioli PA, Amiel E, Schaefer TM, Connolly JE, Wira CR, Guyre PM. Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract. *Infect Immun*. 2004; 72(10): 5799-5806.
40. Pivarsci A, Nagy I, Koreck A, Kis K, Kenderessy-Szabo A, Szeil M, et al. Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human beta-defensin-2 in vaginal epithelial cells. *Microbes Infect*. 2005; 7(9-10): 1117-1127.
41. Schaefer TM, Fahey JV, Wright JA, Wira CR. Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *J Immunol*. 2005; 174(2): 992-1002.
42. Hart KM, Murphy AJ, Barrett KT, Wira CR, Guyre PM, Pioli PA. Functional expression of pattern recognition receptors in tissues of the human female reproductive tract. *J Reprod Immunol*. 2009; 80(1-2): 33-40.
43. Saeidi S, Shapouri F, Amirchaghmaghi E, Hoseinifar H, Sabbaghian M, Sadighi Gilani MA, et al. Sperm protection in the male reproductive tract by Toll-like receptors. *Andrologia*. 2014; 46(7): 784-790.
44. Aboussahoud W, Aflatoonian R, Bruce C, Elliott S, Ward J, Newton S, et al. Expression and function of Toll-like receptors in human endometrial epithelial cell lines. *J Reprod Immunol*. 2010; 84(1): 41-51.
45. Li HWR, Liao SB, Chiu PCN, Tam WW, Ho JC, Ng EH, et al. Expression of adrenomedullin in human oviduct, its regulation by the hormonal cycle and contact with spermatozoa, and its effect on ciliary beat frequency of the oviductal epithelium. *J Clin Endocrinol Metab*. 2010; 95(9): E18-E25.
46. Jorgenson RL, Young SL, Lesmeister MJ, Lyddon TD, Misfeldt ML. Human endometrial epithelial cells cyclically express Toll-like receptor 3 (TLR3) and exhibit TLR3-dependent responses to dsRNA. *Hum Immunol*. 2005; 66(5): 469-482.
47. Lesmeister MJ, Jorgenson RL, Young SL, Misfeldt ML. 17Beta-estradiol suppresses TLR3-induced cytokine and chemokine production in endometrial epithelial cells. *Reprod Biol Endocrinol*. 2005; 3: 74.
48. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol*. 2009; 21(4): 317-337.
49. Nasu K, Narahara H. Pattern recognition via the Toll-like receptor system in the human female genital tract. *Mediators Inflamm*. 2010; 2010: 976024.
50. Lee YL, Lee KF, Xu JS, Wang YL, Tsao SW, Yeung WS. Establishment and characterization of an immortalized human oviductal cell line. *Mol Reprod Dev*. 2001; 59(4): 400-409.
51. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*. 2001; 25(4): 402-408.
52. Gregorczyk KP, Krzyzowska M. Innate immunity to infection in the lower female genital tract. *Postepy Hig Med Dosw (Online)*. 2012; 67: 388-401.
53. Itoh H, Nasu K, Nishida M, Matsumoto H, Yuge A, Narahara H. Human oviductal stromal fibroblasts, but not oviductal epithelial cells, express Toll-like receptor 4: the site-specific mucosal immunity of the human fallopian tube against bacterial infection. *Am J Reprod Immunol*. 2006; 56(2): 91-101.
54. Watters TM, Kenny EF, O'Neill LA. Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. *Immunol Cell Biol*. 2007; 85(6): 411-419.
55. Ling L, Lee YL, Lee KF, Tsao SW, Yeung WS, Kan FW. Expression of human oviductin in an immortalized human oviductal cell line. *Fertil Steril*. 2005; 84 Suppl 2: 1095-1103.
56. Beier HM, Beier-Hellwig K. Molecular and cellular aspects of endometrial receptivity. *Hum Reprod Update*. 1998; 4(5): 448-458.
57. Classen-Linke I, Alfer J, Hey S, Krusche CA, Kusche M, Beier HM. Marker molecules of human endometrial differentiation can be hormonally regulated under in-vitro conditions as in-vivo. *Hum Reprod Update*. 1998; 4(5): 539-549.
58. von Rango U, Classen-Linke I, Kertschanska S, Kemp B, Beier HM. Effects of trophoblast invasion on the distribution of leukocytes in uterine and tubal implantation sites. *Fertil Steril*. 2001; 76(1): 116-124.
59. Spornitz UM. The functional morphology of the human endometrium and decidua. *Adv Anat Embryol Cell Biol*. 1992; 124: 1-99.
60. Yeaman GR, Guyre PM, Fanger MW, Collins JE, White HD, Rathbun W, et al. Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. *J Leukoc Biol*. 1997; 61(4): 427-435.
61. Givan AL, White HD, Stern JE, Colby E, Gosselin EJ, Guyre PM, et al. Flow cytometric analysis of leukocytes in the human female reproductive tract: comparison of fallopian tube, uterus, cervix, and vagina. *Am J Reprod Immunol*. 1997; 38(5): 350-359.
62. Hunt JS. Immunologically relevant cells in the uterus. *Biol Reprod*. 1994; 50(3): 461-466.
63. Qiu F, Cui Z. CD4+ T helper cell response is required for memory in CD8+ T lymphocytes induced by a poly(I:C)-

- adjuvanted MHC I-restricted peptide epitope. *J Immunother.* 2007; 30(2): 180-189.
64. Welters MJ, Bijker MS, van den Eeden SJ, Franken KL, Melief CJ, Offringa R, et al. Multiple CD4 and CD8 T-cell activation parameters predict vaccine efficacy in vivo mediated by individual DC-activating agonists. *Vaccine.* 2007; 25(8): 1379-1389.
  65. Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem.* 2004; 279(17): 17079-17084.
  66. Yu L, Wang L, Chen S. Endogenous Toll-like receptor ligands and their biological significance. *J Cell Mol Med.* 2010; 14(11): 2592-2603.
  67. Frantz S, Kelly RA, Bourcier T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *J Biol Chem.* 2001; 276(7): 5197-5203.
  68. Wan T, Zhou X, Chen G, An H, Chen T, Zhang W, et al. Novel heat shock protein Hsp70L1 activates dendritic cells and acts as a Th1 polarizing adjuvant. *Blood.* 2004; 103(5): 1747-1754.
  69. Laflamme J, Akoum A, Leclerc P. Induction of human sperm capacitation and protein tyrosine phosphorylation by endometrial cells and interleukin-6. *Mol Hum Reprod.* 2005; 11(2): 141-150.
  70. Rodriguez-Martinez H, Kvist U, Ernerudh J, Sanz L, Calvete JJ. Seminal plasma proteins: what role do they play?. *Am J Reprod Immunol.* 2011; 66 Suppl 1:11-22.
  71. Aflatoonian R, Fazeli A. Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. *J Reprod Immunol.* 2008; 77(1): 7-13.
  72. Sioud M. Innate sensing of self and non-self RNAs by Toll-like receptors. *Trends Mol Med.* 2006; 12(4): 167-176.
  73. Yao XD, Fernandez S, Kelly MM, Kaushic C, Rosenthal KL. Expression of Toll-like receptors in murine vaginal epithelium is affected by the estrous cycle and stromal cells. *J Reprod Immunol.* 2007; 75(2): 106-119.
  74. Vahdat F, Hurtgen JP, Whitmore HL, Seguin BE, Johnston SD. Decline in assayable progesterone in bovine plasma: effect of time, temperature, anticoagulant, and presence of blood cells. *Am J Vet Res.* 1981; 42(3): 521-522.
  75. Gresack JE, Frick KM. Effects of continuous and intermittent estradiol treatments on memory in aging female mice. *Brain Res.* 2006; 1115(1): 135-147.
  76. Gresack JE, Frick KM. Post-training estradiol enhances spatial and object memory consolidation in female mice. *Pharmacol Biochem Behav.* 2006; 84(1): 112-119.
  77. Karagenc L, Lane M, Gardner DK. Oestradiol, cyclodextrin-encapsulated 17beta-oestradiol and the oestradiol solubilizer 2-hydroxypropyl-beta-cyclodextrin all impair preimplantation mouse embryo development. *Reprod Biomed Online.* 2004; 9(3): 280-286.
  78. Koster F, Felberbaum R, Finas D, Wunsch K, Schulz C, Diedrich K, et al. Progesterone and estradiol enhance lipid mediated transfection of Sk-Br-3 mammalian cancer cells. *Int J Mol Med.* 2002; 9(6): 617-620.
  79. Rucker B, Pochmann D, Furstenau CR, Carneiro-Ramos MS, Battastini AM, Barreto-Chaves ML, et al. Effects of steroid hormones on synaptosomal ectonucleotidase activities from hippocampus and cortex of adult female rats. *Gen Comp Endocrinol.* 2005; 140(2): 94-100.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.